CD6: expression during development, apoptosis and selection of human and mouse thymocytes

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Abstract

CD6, a 130-kDa surface glycoprotein, is expressed primarily on cells of T lineage. A co-stimulatory role for CD6 in mature T cells has been shown, but the function of CD6 during thymocyte development is unknown. Since CD6 ligands are expressed on thymic epithelium, their interactions with CD6 could be important in thymic selection. In this report we show that CD6 is developmentally regulated in human and mouse thymocytes, and further demonstrate that increase in the level of CD6 expression correlates with expression of the selection marker CD69. We also show that activation via CD2 induces CD6 expression on mature human thymocytes and on a subset of immature human thymocytes that are resistant to apoptosis. In human and mouse thymocytes that express heterogeneous TCR, CD6 increases occur as double-positive thymocytes are selected to a single-positive stage. In contrast, in thymocytes from TCR transgenic mice, CD6 is barely increased following selection, suggesting that as functional avidity increases, requirements for CD6-dependent co-stimulation decrease. Taken together, these results indicate that during thymic development CD6-dependent signals may contribute both to thymocyte survival, and to the overall functional avidity of selection in both man and mouse.

Introduction

Thymocyte selection is an ordered process during which T cells capable of mediating protective immunity are generated (1,2). To be selected, immature thymocytes must express TCR capable of interacting with antigen-presenting cell (APC)±MHC±antigen complexes that can then transduce signals favoring selection (2). This occurs in a manner appropriate for selection in a minority of thymocytes (3). A majority of thymocytes die due to insufficient functional avidity for APC±MHC±antigen complexes ('death by neglect') (2–4), while those thymocytes with excessive functional avidity suffer activation-induced cell death (AICD) (5,6). Therefore, successful thymocyte selection requires optimal functional avidity so that the total signal transduced to immature thymocytes is sufficient to avoid death by neglect, but not so great as to induce AICD (2–6).

A variety of co-stimulatory molecules have been described that may alter the overall functional avidity of selection either by affecting TCR-dependent signals or by inducing signals independent of the TCR. CD5 is an example of a co-stimulatory protein that is important during thymocyte selection (7–10). Because CD5 knockout mice were initially reported to have normal T cell development, delineation of the importance of CD5 in thymic selection was delayed (10). However, studies of antigen-restricted thymocytes from TCR

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transgenic (Tg) mice that lack either CD5 or that lack a portion of the TCR required for signaling have highlighted the importance of CD5 in thymocyte development. These studies show that on mature single-positive (SP) thymocytes, CD5 surface expression directly parallels the avidity and/or signaling intensity of the positively selecting ligand (7). Furthermore, absence of CD5 increases response to TCR-dependent signals and CD5-dependent inhibition of selection appears, in part, by decreasing efficiency of TCR-mediated signaling (7). Taken together, the data have led to identification of CD5 as a negative regulator of selection (7–10) and it has been proposed that similar proteins homologous to CD5, such as CD6, may also play a role in thymocyte selection (11).

CD6 is located 5¢ to the CD5 gene and, like CD5, is a member of the macrophage scavenger receptor family of proteins (11–16). CD6 is a co-stimulatory protein that contains three extracellular cysteine-rich domains, which may differ from each other, both in ligand binding and signaling function(s) (17–23). The CD6 ligand, CD166 [activated leukocyte cell adhesion molecule (ALCAM)], is expressed on thymic epithelium and mediates the CD6-dependent binding of thymocytes to thymic epithelium via a membrane proximal extracellular scavenger receptor domain of CD6 (22–25).

CD6 expression increases with thymocyte maturation and is up-regulated in vitro by anti-CD2 mAb. These anti-CD2 mAb activate both thymocytes and mature T cells, but alter the level of CD6 expression only in thymocytes (26). To delineate the function of CD6 in thymocyte development we characterized the expression of CD6 on developing human and mouse thymocyte subsets, determined to what degree human and mouse thymocyte CD6 is similarly regulated, and obtained evidence regarding a possible role for CD6 in positive selection. We also analyzed whether CD6 expression correlated temporally with the appearance of other cell surface molecules that are reported to mark thymocyte selection, determined whether there is evidence for involvement of CD6 in cell survival and, finally, assessed whether CD6-dependent signals contribute to functional avidity during positive selection.

Results of our studies suggested that expression of CD6 was an important contributor to immature thymocyte survival in both man and mouse. Furthermore, CD6-dependent signals may also be important in co-stimulation of thymocytes, allowing them to survive particularly when the overall functional avidity of thymocytes for APC–MHC–antigen complexes is low. This could have potential implications for autoimmunity.

### Methods

#### Human thymus

Human thymic biopsy specimens derived from children <2 years of age undergoing corrective cardiac surgery were obtained with the approval of the Institutional Review Boards of the University Hospitals of Cleveland and the University of Michigan Hospital.

#### Mice

Colonies of C57Bl/6 (H-2b) and BALB/c (H-2d) mice are maintained in the CWRU animal facility. Ovalbumin (OVA) TCR

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The sources of the reagents used are as follows: (1) gift of E. Reinherz and S. Schlossman, (2) American Type Culture Collection (Rockville, MD), (3) Ancell (Bayport, MN), (4) PharMingen, (5) Biosource (Camarillo, CA), (6) produced in Parnes’ laboratory (15), (7) gift of A. Levine (27), (8) Gibco/BRL (Rockville MD), (9) Caltag (Burlingame, CA), (10) Sigma and (11) Coulter Immunotech (Miami, FL). Fluorochromes (not defined in text): U = unconjugated, F = FITC, PE = R-phycoerythrin; B = biotin; R670 = covalent conjugated Cy5 and PE.
323–339 Tg mice are maintained by A. Levine (gift of K. Murphy) (27). OVA TCR 323±339 Rag-2±/± mice (H-2d), B10.D2 control mice (H-2d) and H-Y TCR Rag-2 ±/± mice (H-2b) (28) were purchased from Taconic (Germantown, NY).

Isolation and tissue culture of thymocytes

Thymocyte single-cell suspensions were generated by gently disrupting the tissue, followed by gradient centrifugation as previously described (26). Red cells were lysed using NH4Cl (15). Human thymocytes were cultured in RPMI supplemented with 10% FBS (Gibco/BRL, Gaithersburg, MD), 2 mm L-glutamine, and 100 U/ml penicillin and 100 μg streptomycin (complete media). For mouse thymocytes, 5.5 x 10^-5 M 2-mercaptoethanol was added to complete media (15). Thymocytes were cultured in six- or 24-well tissue culture dishes (Falcon, Lincoln Park, NJ) for 6–48 h at 37°C with 5% CO2.

Antibodies, fluorochromes, chemicals and apoptosis detection reagents

The antibodies used are shown in Table 1. Staining was performed by incubating cells with saturating concentrations of mAb for 30 min at 4°C. Mouse cells were pre-incubated with Fc block (CD16/CD32) (PharMingen, San Diego, CA) for 10 min at room temperature. Cells were washed twice with SMEM supplemented with 2% FBS as previously described (17,18). When biotin conjugates were used for staining, staining was performed using PBS supplemented with 0.5% w/v biotin-free BSA (Sigma, St Louis, MO) instead of SMEM with 2% FBS. Cells were fixed using 1% formaldehyde and stored at 4°C in the dark until analysis. For Annexin V, propidium iodide (PI) and 7-aminoactinomycin D (7-AAD) staining, cells were analyzed within 30–60 min of completion of the staining.

For overnight culture, anti-CD2/CD2R mAb, anti-T112 and anti-T113 were used as ascites at 1:500 and 1:250 dilutions respectively, anti-CD3 mAb OKT-3 at 1:500 dilution, and purified Ms IgG1 at 2 mg/ml (29,30). Chemicals included phorbol myristate acetate used at 1 ng/ml (Sigma) and dexamethasone used at 1 x 10^-5 M (American Regent, Shirley, NY) (26,31). To sort thymocytes, thymocytes stimulated overnight were labeled with UMC6-FITC (19). mAb used for sorting were washed in Millipore filters that retain proteins >30,000 kDa (Millipore, Bedford, MA) to eliminate azide using PBS with Ca²⁺ and Mg²⁺.

Flow cytometry and cell sorting

Analysis of labeled cells was performed using either (i) an Epics XL-MCL (Beckman Coulter, Miami, FL) equipped with a 488 nm argon ion laser operating at 15 mW of power and System II (version 3.0) acquisition software for data collection.

Fig. 1. Human CD6 expression is developmentally regulated. CD4, CD8 and CD6 expression was measured on freshly isolated human thymocytes. A representative experiment is shown. A double-color histogram for CD4 and CD8 (A) shows the thymocyte populations on which CD6 was measured. R1 (total thymocytes) is composed of the following subsets: CD8 SP (quadrant 1), CD8 DP (quadrant 2), CD8 DN (quadrant 3) and CD4 SP (quadrant 4). The percentage of total thymocytes within each of the quadrants is as indicated. Single-color histograms of CD6 expression (B–E, solid line) show that CD6 expression is highest on CD4 SP > CD8 SP > DP > DN thymocytes. Background staining is as indicated in (B) (dashed line). CD4 SP thymocytes are composed of a CD6intermediate and CD6high subset. The MCF for CD8 SP thymocytes is in between those of the CD4intermediate and CD4low SP subsets.
or (ii) a BD LSR system (Becton Dickinson, San Jose, CA) equipped with a 633 nm helium neon laser operating at 17 mW of power, a 488 nm argon laser operating at 20 mW of power, a UV laser that was not used and CellQuest acquisition software for data collection. Fluorescence emission for FITC, phycoerythrin, Red 670, allophycocyanin and PerCP were collected with optical band pass filters of 525, 575, 675, 660 and 670 nm long pass respectively. Cell sorting experiments were done on an Elite ESP cell sorter (Beckman Coulter), equipped with an 15 mW argon ion laser (488 nm) and a 10 mW helium-neon (HeNe) laser (633 nm). List-mode data collected using Elite acquisition 4.01 software.

The Coulter instruments were calibrated daily using Flow Check beads (Beckman Coulter) and pre-programmed computer settings with fixed voltages to align the instruments, verify full CVs and assure equivalent mean channel fluorescence of controls run on different days. For the BD LSR Align Flow 2.25 μm beads were used instead (Molecular Probes, Eugene, OR). Live cells were gated on the basis of forward and side scatter characteristics. When staining was performed using Annexin V–FITC to tag apoptotic cells, dead cells were excluded from analysis with the viability dye (7-AAD) (Molecular Probes). 7-AAD fluorescence was collected with a 675 nm optical band pass filter. At least 10,000 gated events were acquired per sample in a list-mode file. Off-line data analysis was done with WinList PC version 5.0 software (Verity Software House, Topsham ME).

Apoptosis ELISA

ELISAs were performed using a Cell Death Detection ELISA Plus kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. As breaks in the DNA strand occur early in apoptosis, histone proteins are exposed and are available to bind to the ELISA substrate (anti-histone antibody). Therefore, in this ELISA assay, the amount of histone proteins measured reflects the number of apoptotic cells. Cells (10,000) were lysed according to the protocol provided with the kit and an amount of lysate corresponding to 1000 cells plated in triplicate in 96-well flat-bottom plates. Results were read in an ELISA plate reader (MR 5000; Dynatech, Guernsey, UK).

Results

CD6 protein expression is developmentally regulated in human thymocytes

Using flow cytometry we measured CD6 expression on human and mouse thymocyte subsets, and characterized how CD6 expression varies with the stage of thymocyte development in both man and mouse (Figs 1 and 2). An increase in CD6

Fig. 2. Mouse CD6 expression is developmentally regulated. CD4, CD8 and CD6 expression was measured on thymocytes from a C57Bl/6 mouse. A representative experiment is shown. A double-color histogram for CD4 and CD8 (A) shows the thymocyte populations on which CD6 was measured. R2 (total thymocytes) is composed of the following subsets: CD8 SP (quadrant 2), CD4CD8 DP (quadrant 3), CD4CD8 DN (quadrant 4) and CD4 SP (quadrant 5). The percentage of total thymocytes within each of the quadrants is as indicated. Single-color histograms of CD6 expression (B–E, solid line) show that, as in human thymocytes, CD6 expression in mouse thymocytes is highest on CD4 SP > CD8 SP > DP > DN. Background staining is as indicated in (B) (dashed line).
expression was observed as thymocytes progressed through development from being immature CD4 CD8 double-negative (DN) cells, to immature CD4 CD8 double-positive (DP) cells, and finally to mature CD4+ and CD8+ SP thymocytes (Figs 1 and 2). In man, the majority of DP thymocytes express CD6 at intermediate levels (Fig. 1C), between that of immature and mature thymocytes (Fig. 1B, D and E). Single-color histograms of CD6 expression revealed that in man, a minor subset of DP thymocytes express CD6 at levels comparable to those expressed by SP mature thymocytes (Fig. 1B-E). A small subset of mouse DP thymocytes expresses CD6 at levels clearly above background (Fig. 2C), whereas the majority of SP thymocytes from both mice and men express high levels of CD6 (Figs 1D and E and 2D and E). We also observed that CD4 SP mature human thymocytes contain a subpopulation of SP thymocytes with intermediate levels of CD6 (CD6intermediateCD4SP). These CD6intermediateCD4SP thymocytes are presumed, but not proven, to be more mature than CD6high SP thymocytes. Since CD6 surface expression is lower on resting peripheral blood T cells than on mature thymocytes, it seems logical that down-regulation of CD6 could begin prior to thymic emigration (26) (and our unpublished data). In mice, a CD6low CD4SP subset is present, but is less distinct than that seen in human thymus. Additionally, a CD6low SP subset was more evident among mouse thymocytes than in human thymocytes (Figs 1 and 2).

Developmentally restricted regulation of thymocyte CD6 by CD2: CD2-dependent signals regulate expression of CD6 on thymocytes

We have previously reported that human thymocyte CD6 expression was increased in vitro by cross-linking CD2 with combinations of anti-CD2 antibodies, whereas CD6 expression in mature lymphocytes remained unaffected by perturbation of CD2 (26). To understand the developmental stages at which thymocyte CD6 levels are tunable, we stimulated single-cell human thymocyte suspensions in vitro with mAb directed against CD3 or against both CD2 and CD2R. Cross-linking CD3 and CD2 using either anti-CD3 or anti-CD2 + anti-CD2R mAb can activate T cells (29). For comparison control conditions, we used Ms1gG1 or media alone. In both the control group and the anti-CD3-stimulated group, staining with anti-CD4 and anti-CD8 mAb showed the predicted distributions of thymocytes into immature and mature subsets based on past studies (32). Notably, the group stimulated via CD2 demonstrated increases in absolute numbers of DP thymocytes despite the observation that culture with anti-CD2 mAb transiently modulates CD4 from the surface of DP thymocytes (total thymocyte number in CD2-treated group was increased 50% compared with control) (Fig. 3 and data not shown). Thus, a portion of those cells shown in Fig. 3(B, quadrant 1) are DP thymocytes in which CD4 has been modulated from the surface following perturbation of surface CD2 (32).

Analysis of CD6 expression showed that the majority of CD4 CD8 DP thymocytes in the CD2-stimulated group expressed CD6 at higher levels than the CD3-stimulated or control thymocytes (Fig. 3E and F, and data not shown). A >1.5-fold increase in CD6 mean channel fluorescence (MCF) was observed. In contrast to increases in CD6 expression (Fig. 3G), we observed decreases in CD4 expression in a subpopulation of DP thymocytes (Fig. 3B). In these studies, cross-linking of CD2 also increased CD6 on mature SP thymocytes [MCF 86.80 (anti-CD2/CD2R) versus 61.64 (medium) for CD4 SP, Fig. 3(G)], but not on peripheral blood lymphocytes (26). Taken together, the data showed that regulation of CD6 by CD2 in T cells is developmentally restricted.

CD6 as a marker of positive selection

Our observation of high levels of CD6 expression on a subset of both DP and SP thymocyte led us to hypothesize that increases in CD6 protein precede positive selection. Since it is already established that CD6 expression occurs on DP thymocytes near the time of selection, we reasoned that if CD6 marks selection, then CD6, like CD69, should also be increased on pre-selected DP thymocytes (33,34). CD6 expression was observed on ~15–23% of mature human thymocytes and up to 30% of mature mouse thymocytes (Fig. 4A and B). We observed that CD6 is expressed at high levels on the majority of CD69+ thymocytes in man and mouse (Fig. 4A and B). CD6 MCF is ~3-fold higher on human and up to 20-fold higher on mouse CD69+ than on CD69− thymocytes (Fig. 4A and B).

To explain the observation that some thymocytes were CD6high+, but lacked CD69 surface expression, we hypothesized that the CD6highCD69− thymocytes had already undergone selection and had recently down-regulated CD69 (Fig. 4A and B). For human and mouse studies, we determined the percentage of CD69+ thymocytes in a given sample and then analyzed an identical percentage of thymocytes that expressed the highest levels of CD6: we discovered that the numbers of DP and SP thymocytes in both the CD69+ thymocyte subset and the CD6highsubset were the same (data not shown). Since CD69 expression has been described on thymocytes undergoing both negative and positive selection (3), we tested whether the CD69+CD6+ thymocytes we detected were viable or apoptotic. We stained human thymocytes with either CD69 or CD6, Annexin V (binds to apoptotic cells) and either PI or 7-AAD (excludes dead cells). The data showed that the majority of CD69+ cells are viable, not apoptotic (Fig. 4C). These data are consistent both with studies showing that most CD69+ human thymocytes survive and differentiate in fetal organ cultures in vitro, and with studies showing human thymocytes in the thymic cortex undergo significant apoptosis, whereas few mature CD3high thymocytes undergo apoptosis (34,35).

Our data suggests that a CD6highCD69− thymocyte subset is found both immediately prior to and following selection. CD6lowCD69− thymocytes are most likely DP thymocytes that have not yet acquired CD69. Triple staining of human thymocytes for CD4, CD8 and CD69 expression showed that CD69− thymocytes are comprised primarily of DP thymocytes, whereas CD69+ thymocytes contain more SP (presumed to be recently selected) than DP thymocytes (presumed to be undergoing positive selection) (Fig. 4D). We hypothesize, but have not directly shown, that the CD69−CD6− subpopulation (3–4%, Fig. 4A and B) of non-dead thymocytes is composed of CD69−AnnexinV+ apoptotic thymocytes (3.15%, Fig. 4C).

CD6-dependent signals have been implicated in protecting B-CLL cells from antigen-mediated apoptosis, but not other
Fig. 3. Human thymocyte CD6 expression is induced by a combination of anti-CD2 mAb in vitro. Human thymocytes were stimulated overnight with anti-CD2 mAb, anti-CD3 mAb, or control conditions using MsIgG1 or medium without antibody. A representative experiment in which thymocytes were stained for CD4, CD8 and CD6 expression is shown. (A–D). Expression of CD4 (x-axis) and CD8 (y-axis) is depicted for R1 (total thymocytes) as in Fig. 1. Distinct CD4highCD8+ versus CD4mediumCD8+ subpopulations can be distinguished within the DP subset (Fig. 2B) after stimulation via CD2, but not in control cultures with media alone (A), MsIgG1 (C) or anti-CD3 mAb (D), or control medium and MsIgG1 (data not shown). (E and F). CD6 staining on total thymocytes (R1, all quadrants) and DP thymocytes (R1, quadrant 2) are shown. CD6 expression is increased in thymocytes treated with anti-CD2 mAb to a greater extent than in those treated with anti-CD3 mAb (F). A dotted vertical line is drawn through the peak of the single-color CD6 histogram of thymocytes stimulated via CD3 (2F). A vertical line is placed at the same position in the single-color CD6 histogram from thymocytes stimulated via CD2/CD2R to more easily illustrate the relative increase in CD6 expression that occurs after stimulation with anti-CD2 mAb (Fig. 2E). (G). The MCF of CD6 expression for CD4 SP thymocytes is shown in table form along with the MCF for DP thymocytes (R1, quadrant 2). (H) The percent increase in CD6 MCF due to cross-linking of CD2 or CD3 was compared to stimulation with phorbol myristate acetate, which is known to increase CD6 expression, relative to media control. The relative increase in total thymocyte CD6 MCF was calculated by dividing the absolute MCF (sample – background) for each condition by the absolute MCF of thymocytes cultured in media alone and multiplying by 100. Not every condition was tested in each experiment. Results are expressed as the % increase in MCF ± SEM.
types of apoptosis (36). Since resistance to apoptosis is fundamental to accomplishing positive selection and our data suggested that increases in CD6 mark selection, we next hypothesized that CD6 might be important for survival of developing thymocytes. Therefore, to determine if CD6 is involved in cell survival, we compared CD6 surface expression on viable and apoptotic thymocytes.

Expression of CD6 was associated with thymocyte resistance to apoptosis

To study the relationship between CD6 expression and apoptosis, an in vitro model in which CD6 levels could be manipulated was desirable. Since CD6 is regulated in thymocytes via CD2-dependent signals and cross-linking of CD2 may induce T cell apoptosis, we performed in vitro experiments in which thymocyte CD6 could be manipulated using anti-CD2 mAb to cross-link CD2 (32,37,38). We used a variety of conditions that have been previously demonstrated to induce thymocyte apoptosis, including serum withdrawal, culture with dexamethasone, and cross-linking of cell surface receptors such as CD3 and CD2 (31). We hypothesized that CD2-dependent signals could trigger either increases in CD6 expression or apoptosis in a given thymocyte, but that these events would be mutually exclusive.

To test our hypothesis, we cultured thymocytes for 16–24 h with anti-CD2 mAb, dexamethasone or in medium alone (Fig. 5). We then stained thymocytes with Annexin V–FITC, and compared CD6 expression on viable and apoptotic (but not dead) cells using flow cytometry (Fig. 5). The data showed that when thymocytes were subjected to stimuli with the potential to induce apoptosis (especially activating stimuli transduced via CD2), CD6 was expressed at higher levels on the viable thymocytes compared with the apoptotic cells. Thymocytes cultured with dexamethasone showed significantly higher rates of apoptosis than thymocytes cultured either with anti-CD2 mAb or in medium alone.

High CD6 expression correlated with resistance to apoptosis in sorted human thymocytes

To verify that high CD6 expression on thymocytes marks a subset resistant to apoptosis, thymocytes were cultured overnight as described above and were sorted according to CD6 expression (Fig. 6). Cell lysates were used in an ELISA assay to measure the rate of apoptosis in both CD6high and CD6low thymocyte subsets. The data showed that CD6high thymocytes have a lower rate of apoptosis than CD6low thymocytes, especially following stimulation via CD2 (Fig. 6). Differences in apoptosis are not explained by varying proportions of mature and immature thymocytes in the samples.
Taken together, the data suggest that even moderate increases in CD6 level correlate with decreases in the rate of apoptosis. Expression of a Tg TCR decreases requirement for CD6 expression: TCR-Tg thymocytes have minimal increase in CD6 during selection/maturation from DP to SP thymocyte. The observed pattern of CD6 expression also raises the possibility that CD6 may enhance the overall functional avidity between thymocytes and APC–MHC–antigen complexes, increasing the overall number of immature thymocytes selected. To pursue this issue we characterized the pattern of CD6 expression in thymocytes derived from two different antigen-specific TCR-Tg mice. We used OVA TCR mice to examine selection of CD4 SP thymocytes and H-Y TCR mice to examine selection of CD8 SP thymocytes (27,28). If CD6 is important for increasing the overall functional avidity of the T cell for MHC–antigen complexes, then the presence of a Tg TCR that boosts overall functional avidity during selection might decrease the need for co-stimulation or adhesion via CD6.

Since the overall functional avidity of selection is increased by the presence of Tg TCR specific for OVA peptide 323–339, we hypothesized that OVA TCR thymocytes would require less co-stimulation via CD6 than thymocytes with heterogeneous TCR to achieve maturation to a CD4 SP status. We compared
CD6 expression in OVA TCR-Tg mouse thymocytes (H-2d) to wild-type thymocytes (H-2d) (Fig. 7). We confirmed that CD3<sup>high</sup> thymocytes contain almost exclusively mature SP thymocytes and that the CD3<sup>dull</sup> subset is comprised of immature DP thymocytes (data not shown). We then analyzed OVA TCR or BALB/c thymocytes for expression of OVA TCR, CD3, and CD6. The data show that a smaller subset of immature CD3<sup>dull</sup> thymocytes from OVA TCR mice and the major subset of immature CD3<sup>dull</sup> thymocytes in wild-type controls express similar levels of CD6 (R7; Fig. 7A, right upper and B right lower). A subset of DP and SP thymocytes from these OVA TCR mice expressed low levels of CD6 (R6, left peak; R7, left peak). Less extensive CD6 up-regulation was required for positive selection of OVA TCR thymocytes than was seen with wild-type thymocytes (Fig. 7).

To determine whether diminished up-regulation of CD6 also accompanies selection of CD8 SP T cells in the presence of a Tg TCR, thymocytes from H-Y (male antigen) female mice (H-2b) were used (28). These mice lacked Rag-2 and, thereby, expressed only H-Y TCR. The data show that both H-Y TCR DP and CD8 SP thymocytes express less CD6 than do thymocytes from either syngeneic C57Bl/6 (H-2b) or allogeneic BALB/c (H-2d) mice at a similar maturational stage. Furthermore, in contrast to the increases in CD6 expression we described in thymocytes from wild-type mice and from humans, only a minimal increase in CD6 expression level was observed as H-Y TCR thymocytes progress from being DP to being CD8 SP (Fig. 8A).

Since the two models of thymocyte selection (OVA TCR and H-Y TCR) we used differ in their potential to rearrange their TCR, we wanted to determine if increases in CD6 expression were attenuated in OVA TCR that lacked Rag-2. Therefore, we compared CD6 levels on OVA TCR Rag-2<sup>−/−</sup> mice (H-2d) to control B10.D2 (H-2d) mice (Fig. 9). In these thymocytes from OVA TCR Rag-2<sup>−/−</sup> mice, CD6 expression was similar on immature (CD3<sup>dull</sup>) and mature (CD3<sup>high</sup>) thymocytes, whereas CD6 was increased on SP to DP thymocytes from control B10.D2 mice.

The data from mouse thymocytes show that developmentally specific CD6 up-regulation was reduced in the presence
of a Tg TCR for selection of either CD4 or CD8 SP thymocytes, consistent with a model in which CD6 is more important for co-stimulation when the overall functional avidity of selection is low.

**Discussion**

Human CD6 has been identified and cloned, but its precise function(s) in both developing and mature T cells is unknown (15,16,39,40). Regulated CD6 expression accompanied by expression of CD6 ligands (CD6L) on thymic epithelium strongly suggest a specific role for CD6 in thymocyte development. Experiments performed in thymocytes from man and mouse and presented in this report suggest that physiologic CD6–CD6L interactions might act to: (i) send anti-apoptotic signals to immature thymocytes, and (ii) increase the functional avidity of binding between MHC–antigen complexes on thymic APC and thymocytes to levels sufficient to enhance positive selection without inducing negative selection.

**Role of CD6-dependent signals in positive selection**

Evidence that CD6, like CD69, is also a marker for selection is strongly suggested by data showing that increases in CD6 expression are accompanied by expression of CD69 in human and mouse thymocytes. Data presented in this study, showing that stimulation via anti-CD2 increases CD6 expression as well as thymocyte numbers, together with previously published reports (41–47) provide strong support to our hypothesis that CD6, like CD2, is involved in thymocyte selection. The inverse correlation between thymocyte CD6 expression and rate of apoptosis favors a role for CD6-dependent effects on the balance between pro- and anti-apoptotic proteins in developing thymocytes. These observations are consistent with data showing that cross-linking of CD6 in B-CLL cells appears to protect against antigen-mediated apoptosis and induces the expression of anti-apoptotic proteins (36,42,48). We suggest that activation of DP thymocytes occurs in vivo due to antigen recognition accompanied by both CD2–CD2L interactions (e.g. LFA-3, CD58 in humans) and other co-stimulatory interactions while thymocytes are CD6low. Activated thymocytes first up-regulate CD6 and then express CD69 or else undergo apoptosis. DP thymocytes with increased surface CD6 bind to CD6L on thymic epithelium at increased frequency and these interactions may help to trigger development from a DP to a SP stage. Selected SP thymocytes then down-regulate CD69 and eventually down-regulate CD6.
Role of CD6-dependent signals in functional avidity

Results of our studies showed that CD6 was down-regulated in some SP thymocytes from humans and wild-type mice, and we hypothesize that this occurred at a time when CD6-dependent co-stimulation was no longer required because the selection process was complete. In H-Y TCR-Tg thymocytes, absolute levels of CD6 were lower in both DP and SP thymocytes than in wild-type mice. In OVA TCR mice the most obvious aberration in CD6 expression was that CD6 was only minimally increased during transition from DP to SP stage. This could be due to limited augmentation of CD6 in SP thymocytes from Tg mice because the Tg TCR acts to increase overall functional avidity of selection, thereby reducing relative requirements for CD6-dependent signals. Differences between the various TCR-Tg mice (OVA versus H-Y TCR) may relate to the relative functional avidities of the respective TCR for APC-MHC-antigen complexes (7,44,45,49). However, further studies may be necessary to definitively confirm or negate this assumption.

In human thymocytes we observed a close association between CD4 and CD6 levels in CD4 SP mature thymocytes. It is conceivable that interactions between thymocyte CD6 and thymic epithelial CD6 ligands contribute to the positive selection of CD4 SP thymocytes in a manner similar to that of CD4-MHC class II interactions (49). Whether increases in CD6 occur in experimental systems that lack critical co-stimulatory proteins (and thereby have less positive selection) is not yet known.

We have previously demonstrated that CD6 is increased in mature T cell clones induced with drugs to become autoreactive and that anti-CD6 mAb inhibit reactivity with self-APC (18). Modest increases in CD6 were found to be functionally important in these studies (18). In mature T cells, excess functional avidity with self-antigen in APC-MHC-antigen complexes results in autoimmunity (50). In the periphery, excesses in CD6-dependent co-stimulation may augment functional avidity with self-antigen, predisposing to autoimmunity. Our new data suggest that human and mouse CD6 both function similarly during thymic selection, a process in which functional avidity is critical.

Our new studies of CD6 in human and mouse thymocytes suggest that CD6-dependent signals tune selection in favor of thymocytes that have otherwise have insufficient functional avidity for MHC-antigen complexes to compete for selection. This model of CD6 requires additional proof using in vivo experimental systems. Nevertheless, our studies suggest that increases in CD6 expression facilitate co-stimulatory signaling, increase overall functional avidity and oppose apoptosis, in the end favoring thymocyte selection and maturation. Additionally, our data raise the possibility that supra-physiologic expression of CD6 might increase functional avidity, the consequences of which could either be increased positive functional avidity or less positive selection.

Fig. 9. Thymocytes from OVA TCR Rag-2+/− Tg mice showed no increase in CD6 expression following selection. Thymocytes from OVA TCR DO 11.10 (H-2d) or control B10.D2 mice were stained for CD3 and CD6 expression. A representative experiment is shown. The CD3+ subset (R6, middle) in both mice is composed of SP thymocytes, whereas the CD3− subset (R7, middle) is composed of CD4 CD6 DP thymocytes (data not shown). Levels of CD6 are comparable on DP (R7) from OVA TCR (A, right upper) and control mice (B, right lower). As thymocytes mature, CD6 is increased on CD3+ (R6) thymocytes from control mice (B, right lower) more than on CD3+ (R6) thymocytes from OVA TCR mice (A, right upper). The antibodies and developing reagents used in these figures include CD4-allophycocyanin, CD8-PerCP, CD3-FITC, CD6-unconjugated, goat anti-rabbit-biotin and streptavidin-phycoerythrin.
selection (with potential for autoreactivity) or even negative selection, depending on the avidity of a specific TCR for MHC-antigen. Demonstration that human and mouse CD6 have similar function(s) enhances the probability that use of models such as Tg mice that overexpress CD6 and CD6 ‘knockout’ mice will be helpful in future evaluations of the physiologic function(s) of CD6.

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Abbreviations
AAD 7-aminomethicyn D
AICD activation-induced cell death
APC antigen-presenting cell
CD6L CD6 ligand
DN double negative
DP double positive
MCF mean channel fluorescence
OVA ovalbumin
PPI propidium iodide
SP single positive
Tg transgenic

References


